Immunology of onchocerciasis III. Antibody mediated response to *O. volvulus* and its relationship to complement activation

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Inmunologia de la oncocercosis III. Respuesta mediada por anticuerpos y su relación con la activación del complemento

En el suero de 36 individuos indígenas de un territorio amazónico de Venezuela (foco hiperendémico de oncocercosis) se investigó la presencia de anticuerpos específicos contra *Onchocerca volvulus (O. volvulus)*, así como los niveles de IgG, IgA, IgM e IgE, complejos inmunes y componentes del complemento (C3, C4, factores B y H y Cl-inh); mediante ensayos hemolíticos fue también investigada la activación del complemento por la vía clásica y alternativa. Se hallaron niveles significativamente elevados respecto del grupo control, de IgG, IgA e IgE conjuntamente con anticuerpos anti-*O. volvulus*, así como de complejos inmunes especialmente por la técnica del RIA-RAJI e inhibición de las rosetas RAJI, lo que sugiere que dichos complejos inmunes son fijadores del complemento. Los ensayos funcionales demostraron que existía consumo de complemento tanto por la vía alternativa como clásica.

Estos resultados sugieren que en la oncocercosis los complejos inmunes integrados probablemente por anticuerpos anti-*O. volvulus* podrían contribuir a la patogenia de las lesiones vasculares que afectan a la piel y ojos de los pacientes con oncocercosis.

The presence of specific antibodies against *Onchocerca volvulus (O. volvulus)* and the serum levels of IgG, IgA, IgM and IgE, immune complexes and complement components (C3, C4, Factors B and H and Cl-inh) were investigated in serum samples from 36 subjects native of the Amazon territory in Venezuela (hyperendemic focus of Onchocercosis). Haemolytic tests were also used to investigate the complement activation through the classic and alternative pathways. Significatively increased IgG, IgA and IgE serum levels relative to control values together with anti-*O. volvulus* antibodies were found. Moreover, increased levels of immune complexes were observed particularly when the RIA-RAJI and RAJI rosettes inhibition techniques were used, all of which suggest that such immune complexes have the ability to fix complement. Functional tests showed that there was complement consumption through both the classic and the alternative pathways.

These results suggest that in Onchocercosis the immune complexes, probably formed by anti-*O. volvulus* antibodies, may play a role in the pathogenesis of the vasculitis-related lesions responsible for ocular and cutaneous involvement in patients with Onchocercosis.

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INTRODUCTION

Chronic infection with the filarial nematode *Onchocerca volvulus* is associated with pathological changes including the presence of subcutaneous nodules, dermal atrophy, various forms of dermatitis, and disseminated pruritus. More seriously, invasion of the eye by microfilariae can lead to the development of chorioretinal and optic nerve lesions and anterior segment disease impairing vision and ultimately producing blindness.

The underlying causes of the symptoms of onchocerciasis remain unclear, although the similarity of the skin lesions to those seen during Arthus reactions suggests that local formation of immune-complexes may be involved. Large amounts of serum immunoglobulins of different classes are found in onchocerciasis[1,2] and at least some of these are probably complement fixing antibodies directed against skin derived microfilarie[3].

In Venezuela, there exists a recently discovered large endemic area of onchocerciasis in the indigenous Yanomami Amerindians[4]. This populations is heavily parasitized with *O. volvulus* and there is a high prevalence of skin and ocular lesions. In the present study, as part of a research protocol dealing with the immunopathology of Venezuelan onchocerciasis, we have investigated several fluid phase components and their possible relationship to complement activation.

MATERIAL AND METHODS

Patients and controls

This study was conducted on the sera from 36 indigenous Yanomami Amerindians of both sexes, aged 17 to 55 years, living in the area of Parima B (02° 55' N, 64° 14' W) in the Venezuelan Amazonas Territory. This area is within a hyper-

endemic focus of onchocerciasis. The control group of sera was obtained from 200 venezuelan individuals of both sexes, aged 18 to 45 years, selected by a pre-established protocol[5]. None of the control group had ever visited and endemic area of onchocerciasis. A suitable control population of Yanomami Amerindians free of filarial infection has yet to be identified.

Clinical and parasitological examinations

The 38 patients were examined clinically for signs of infection with *O. volvulus.* Multiple cutaneous biopsies were taken[6] to estimate the number of microfilariae present per milligran of skin. A coproparasitological study was made following the protocol of Sapero and Lawless[7].

Preparation of sera

Samples of peripheral venous blood, were allowed to clot for 1 hour at ambient temperature and for a further four hours at 4 °C. The sera were transported on ice in siliconized glass tubes and within the following 12 hours were centrifuged at 500 g for 15 min at 4° C, divided into 0.5 ml aliquots and stored at −70° C.

Reagents

Isotonic triethanolamine buffered saline, pH 7.5, containing 0.15 M calcium chloride, 0.5 mM magnesium chloride and 0.1 % gelatin (TBS) was used to evaluate complement classical pathway (CP). Isotonic veronal buffered saline containing 8 mM EGTA and 2 mM magnesium chloride (VBS-EGTA) was used in assays of alternative pathway (AP). Sheep erythrocytes collected into Alsever's solution were washed three time with TBS, adjusted to an optimal density of 500 at a wavelength of 550 mM and sensitized with rabbit antisheep haemolysin (National Institute of Hygiene, Caracas) while erythrocytes from New Zealand white rabbits were adjusted to the same concentration in VBS-EGTA.

Quantification of immunoglobulins

Concentrations of IgG, IgM and IgA were determined by radial immunodiffusion. Total serum IgE was measured by a radioimmune assay (Phadezym IgE PRIST, Pharmacia, Uppsala, Sweden).

Specific antibody to *O. volvulus*

Specific antibodies to *O. volvulus* were measured by ELISA test following the method of Voller et al[8] with minor modification[9]. A lyophylized crude extract of adult *O. volvulus* isolated from nodules by enzymatic digestion[10,11] was used as the source of antigen. The optimal antigen concentration and serum dilutions were 15 μg/ml and 1:200 respectively. Results were obtained at 450 nM (Dynatech micro-ELISA spectrophotometer) and values over the mean ± 1 s.d. of the control group were considered as positive evidence for specific *O. volvulus* antibodies.

Measurement of circulating immune complexes

Levels of circulating immune complexes (CIC) were measured by three different assays: a Clq-binding assay (Clq-BA)[12]; a Raji cell radioimmune assay (Raji-RIA)[13] and by inhibition of Raji rosettes (I-RR)[14].

Classical pathway haemolytic assays

Complement classical pathway functional activity was explored in both fluid-phase (FPCP) and solid-phase (SPCP) assays. The method of Kent and Fife[15], previously standarized in our laboratory[16] was employed for the FPCP assay. The SPCP assay was performed according to Gewurz et al[17] in 2.2 % (w/v) low gelling temperature agarose in TBS diluted with an equal volume of sensitized sheep erythrocytes. After solidification, 4.5 mm diameter wells were punched and filled with 20ul of the serum under test. The plates were incubated for 12 hours at 4 °C and for a further 6 hours at 37 °C.

Alternative pathway haemolytic assay

The alternative pathway functional activity was also investigated in fluid-phase (FPCP) and solid-phase (SPAP) assays. FPAP assays were performed employing the standarized procedure of Platts-Mills and Ishizaka[18]. Briefly, 0.4 ml of serum dilutions between 1:10 and 1:22.5 in VBG-EGTA were mixed with 0.6 ml of the rabbit erythrocyte suspension and incubated at 60 min at 37 °C in a shaking water bath. The reaction was stopped adding 0.5 ml of VBS-EGTA at 4 °C; the SPAP assay was performed identically to the SPCP assay but using VBS-EGTA as the supporting buffer.

Expression of results from haemolytic assays

Results from the fluid-phase assays (FPCP and FPAP) are expressed in CH_{50} units. Results from solid-phase assays (SPCP and SPAP) are expressed in volumes of haemolysis (Vh) as described by Bourret and Bordet[19] using the formula:

$$Vh = h\,(Dh^2 - Do^2)$$

where Vh = volume of haemolysis, h = gel height, Dh = diameter of ring of haemolysis and Do = diameter of well.

Measurement of complement components

The serum levels of complement components C4, C3, factor B, C1 inhibitor (Clinh) and H were measured by radial immunodiffusion[20] using monospecific antibodies (Atlantic Antibodies, Scarborough, Maine, USA). Levels are expressed in mg % with the exception of protein H which is expressed as a percentage of the level in a pool of normal human serum.

Statistical analysis

Tests of significance were carried out using Student's t-test and pearman rank correlation.

RESULTS

Parasitological studies

Skin-biopsies revealed the presence of microfilariae of *O. volvulus* in the 36 patients with a parasite load between 2.4 and 906 microfilariae per milligram of skin. In the coproparasitological study of the patient group, protozoa and helminths were found in 57 % and 43 % of the population respectively. *Ascaris lumbricoides* was the mayor intestinal helminth identified with

TABLE I. **Mean serum levels on immunoglobulins**

	n	IgG (mg %)	IgA (mg %)	IgE (IU/ml)	IgM (mg %)
Patients	38	3884* ±1366	1154* ±274	4192* ±3236	155 ±25
Controls	100	1300 ±500	200 ±50	200 ±50	100 ±60

* $p < 0.005$

TABLE II. **Levels of circulating immune complexes**

	n	RAJI-RIA (ug/ml)	IRR (%)	Clq-BA (% binding)
Patients	38	107 ± 144*	46 ± 23**	3 ± 4
Controls	200	19 ± 18	5 ± 6°	2 ± 1

° n = 70; * $p < 0.001$; ** $p < 0.005$

TABLE III. **Studies of the complement classical pathway**

	n	FPCP	SPCP	C4	C1 Inh.
Patients	38	118±49*	38±17*	22±10	20±4
Controls	200	200±50	76±6	28±8	19±3

n: numbers; * $p < 0.005$; FPCP: fluid-phase classical pathway; SPCP: solid-phase classical pathway

24 % of the population being infected. None of the control group has detectable protozoan or helminth infections.

Immunoglobulin levels

Total serum levels of IgG, IgA and IgE were appreciably higher in the infected group (table I) while the level of IgM was not significantly different.

Specific *O. volvulus* antibodies

The mean serum values for 98 control sera was 0.38 while all patients studied showed significantly elevated levels of *O. volvulus* antibodies with a mean of 0.903 ± 0.123 and a degree of sensitivity over 80 %.

Circulating immune complexes (CIC)

Abnormally high levels of CIC were found in 100 % of the onchocerciasis group by I-RR assay ($p < 0.005$) and in 50 % of the patients using the Raji RIA ($p < 0.001$) (table II). No significant difference could be detected between the patient and control groups using the Clq-binding assay.

Complement components and activation pathways

The complement system was evaluated both functionally and immunochemically. Known classical (CP) and alternative pathway (AP) activators were employed to assess functional status of the sequence. Simultaneous consumption of both CP and AP was found among the patients group. Furthermore, the complement consumption was better defined in the functional assays (tables III and IV, fig. 1). When paired t test was applied to the analysis of the immunochemical levels, B and C3 were significantly lower when compared to the control group. The levels of C4 and Cl-inh were comparable between the groups (fig. 2).

DISCUSSION

The Sierra Parima of Venezuela is a hyperendemic area of onchocerciasis where more than 70 % of the indigenous Yanomami population is infected with *O. volvulus*[21,22]. Parasite loads as assessed by the frequency of subcutaneous nodules and microfilarodermia, are very high and clinical symptoms of the disease are common.

Little is known regarding the immunopathology of both ocular and skin lesions, even though the histopathology found, often shown similarities to that of an Arthus reaction, which is well known to be mediated by local immune complexes.

We have investigated the humoral compartment of a group of 36 Amerindians, heavily infected with *O. volvulus*, measuring several fluid phase components along with an integrated assessment of the complement activating pathways.

High levels of serum IgG, A and E associated with significantly elevated *O. volvulus* specific antibodies and of CIC levels were found in most of the patients group. Furthermore, the CIC were mainly demonstrated by the RIA-Raji technique and particularly by inhibiting Raji rosette formation[13], thus suggesting the complement fixing properties of these CIC.

TABLE IV. **Studies of the complement alternative pathway**

	n	FPAP	SPAP	C3	B	H
Patients	38	20±6*	34±12	103±25*	22±4*	114±16
Controls	200	30±7	39±9	128±26	33±9	93±17

n: number; * $p < 0.005$; FPAP: fluid-phase alternative pathway; SPAP: solid-phase alternative pathway.

Figure 1. Classical and alternative complement pathways levels in onchocerciasis and controls groups. FPCP: fluid-phase classic pathway; SPCP: solid-phase classic pathway; FPAP: fluid-phase alternative pathway; SPAP: solid-phase alternative pathway. (See material and methods.)

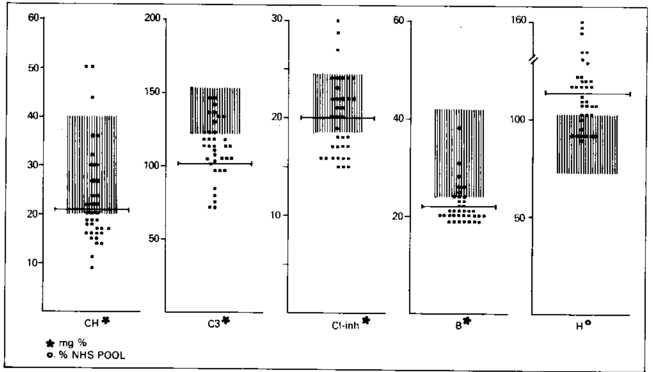


Figure 2. Levels of complement components in both patients and control group.

The observation of activation of both complement pathways in the patients group was remarkable. The significant and simultaneous consumption of both CP and AP has not been shown previously in onchocerciasis. Several mechanism which may be contributing to the activation of the sequence through both pathways should be considered. Thus, interaction of specific anti *O. volvulus* antibodies either with parasites themselves or with substance of parasite origin in the circulation[22] and presumably also in the tissues, may lead to the synthesis of CIC and the subsequent activation of the classical pathway. On the other hand, AP activation may result from interaction with polysulphated acid mucopolysaccharides of parasite origin. Although a precise chemical analysis of the surface of microfilariae of *O. volvulus* is lacking, microfilariae of other species have been shown to contain sulphated mucopolysaccharides[23] and in the case of *Dirofilaria immitis*, these are shed by the parasite and may activate complement directly[24]. The circulating antigen of *O. volvulus* detected by Ouaissi et al[25] may be important in this respect though its chemical nature remains to be determined. Activation of the alternative pathway in onchocerciasis may also be produced by hydrolytic enzymes released by granulocytes. Spry[26] observed that eosinophiles in the blood of patients with tropical filarial eosinophilia were degranulated and hypothesized that the degranulation products could contribute to the symptoms of the disease. In onchocerciasis, free antigens of *O. volvulus* interacting with the large amounts of IgE observed in these patients, probably leads to degranulation of eosinophils in the skin; eosinophils in the skin of infected patients have been found to contain *O. volvulus* antigen (C.D. Mackenzie, personal communication).

Within the context of the results obtained in the present investigation, it may be speculated that the immunopathology of both ocular and skin lesions may be associated with the synthesis of complement fixing immune complexes, with the subsequent and simultaneous activation of both the classical and alternative pathways. The clinical expression in this particular clinical condition is that of a rather limited systemic vasculitis. Nevertheless, new investigations are requiered to assess whether these complexes are related to *O. volvulus* antigens and if other organs (i.e. kidney) may also be involved.

Overall, the immunopathology of onchocerciasis, seems to be associated with the consequences of a complex parasite host interaction, whereby simultaneous antibody and cell mediated immune responses may contribute significantly not only to the generation of the clinical symptoms but also to the impairment of effector responses to new antigenic challenges. Free antigens and other parasitic components, the synthesis of fluid phase components such as immune complexes possibly related to specific *O. volvulus* antibodies, the bioproducts of an activated complement sequence, along with possible modulatory factors of cell behavior may explain the observations obtained when assesing heavily infected amerindians. In immunological studies of infected individuals from the Sierra of Parima, we have already observed that serum and cell factors operating simultaneously can inhibit the blastogenic responses of singenic and allogeneic cells in mixed lymphocyte cultures[27] and the characteristic depression of delayed cutaneous hypersensitivity reactions seen in African generalized onchocerciasis[28] is also exhibited by these patients[29]; this depression appears to be associated with the presence of circulating *O. volvulus* antigen[29]. Lastly, our results offer not only new insight in the pathogenesis of the disease, but a more integrated approach when evaluating a patient with onchocerciasis. This in turn may lead to new forms of therapy which may help prevent such serious complications as blindness.

ACKNOWLEDGEMENTS

This work was supported by CONICIT (Programa de Fortalecimiento de Centros y Proyecto SA-1128), by Ministerio de Sanidad y Asistencia Social and by PROICET-AMAZONAS.

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